IMPACT OF NOREPINEPHRINE ON THE GROWTH AND VIRULENCE OF *CLOSTRIDIOIDES DIFFICILE*

by

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Dedicated to my parents and my husband

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ABSTRACT

Clostridioides difficile infection (CDI) is considered as an urgent threat to the public by CDC, 2019. It causes life-threatening diarrhea and pseudomembranous colitis, mostly in those taking antibiotics or at the end of their antibiotic course. It is also notified as hospital-associated pathogens because one-third of the CDI has occurred in the health care center. Norepinephrine (NE) is a stress-associated neuroendocrine hormone released upon sympathetic stimulation to mediate stress. Gut walls are highly innervated by the sympathetic nervous system. During stress, elevated level of NE released in the GI tract could influence bacterial overgrowth & translocation. It is already known for its role in modulating the behavior of several bacterial pathogens such as *Staphylococcus, Escherichia coli, Salmonella, and Vibrio cholera*. This study aims to evaluate the effect of NE treatment on the growth and virulence of *C. difficile*. Here, we studied the effect of NE on six different *C. difficile* strains isolated from humans. To understand the influence on growth, bacterial culture was treated (+/-)NE (5μM & 50 μM) during their log phase and recorded the density of the cell each time period for constructing the growth curve. In addition, after NE treatment, bacterial cells were taken for further analysis. For investigating the impact of NE on the virulence genes expression, a qPCR reaction was performed along with -RT / noRT control reactions for assessing the RNA sample free from genomic DNA contamination. In the case of growth, higher growth was observed in VPI 10463 at 6 hour time point only, and in strain, NR 49277 significantly stimulated after 6 hours and continued till 8 hours after treatment with 50 μM NE. In strain NR 49282, decreased growth was observed at 7-hour time points after 50 μM NE treatment. But, there was no difference in cell density between control & 5μM NE treated bacterial culture in all strains.

Toxin genes *(tcdA* & *tcdB)*and flagellin gene (*fliC*), were upregulated in NR 49290, NR 49277 & VPI 10463 strains in both concentrations of NE and down-regulated in NR 49282. In strain NR 32888, toxin genes were downregulated while treated with 5μM NE but upregulated after 50μM NE treatment, though fliC was downregulated in both concentrations. In strain NR 32891, *tcdA* was downregulated, but *tcdB* & *fliC* were upregulated after NE treatment in both concentrations. Increased expression in pilin gene, *pilA1* in strain NR 49277, NR 49290, VPI 10463 & NR 32891 in both concentrations was observed. In addition, *pilA3* in NR 49277, VPI 10463 & NR 32891,

and *PilA5* in NR 49277 & NR 49290 showed an upregulation pattern while treated with both concentrations. Modulating this response, it is possible to reduce the pathogenicity of *C. difficile* during medical care & antibiotic use.

CHAPTER 1. INTRODUCTION

1.1 *Clostridioides difficile (C. difficile)*:

Clostridioides difficile (C. difficile), formerly known as *Clostridium difficile*, was first noticed in the fecal matter of a newborn infant and identified as a commensal organism of the intestinal flora in 1935 [1]. It is a Gram-positive, spore-forming bacterium [2]. While discovered, it was given the name "*Bacillus difficilis*." This Latin word *'difficilis'* refers to difficulty giving a brief idea about its isolation difficulty and complications, and slow-growing nature in the prerequisite anaerobic condition [1]. Though it was initially considered non-pathogenic due to its colonization in the neonatal feces [1], it became clear that this bacterium produced toxins later. The name was subsequently changed to *Clostridium difficile* [3]. In 2016, it was reclassified from *Clostridium difficile* to *Clostridioides difficile* to reflect taxonomic differences from *Clostridium butyricum* and related species [4].

1.2 Gut-Brain axis:

In human body structure, the brain is a remarkably complex organ; it is unique for its excellent structure and complexity. The central nervous system (CNS), which consists of the brain and spinal cord, is the main control module and coordinates different body parts. The enteric nervous system (ENS), another autonomic nervous system (ANS), consists of neurons that regulate the function of the gastrointestinal (GI) tract. It is also called the second brain [5,6]. Interestingly, bidirectional communication is maintained between CNS and ENS. This is commonly known as the gut-brain axis (GBA), by which a linkage is maintained between the emotional and cognitive centers of the brain with peripheral intestinal functions [7,8].

The brain creates nerve impulses, stores memories, regulates body movements, and secrets different types of hormones and neurotransmitters. The GI tract maintains food metabolism, nutritional absorption, and microbial communities. In stress conditions such as anxiety, sickness, and depression, different kinds of neurotransmitters are released, which could affect the gut environment. Sometimes, metabolic & nutritional disparity, microbial imbalance, etc., influence brain function. So, the brain and gut influence each other by this reciprocal communication.

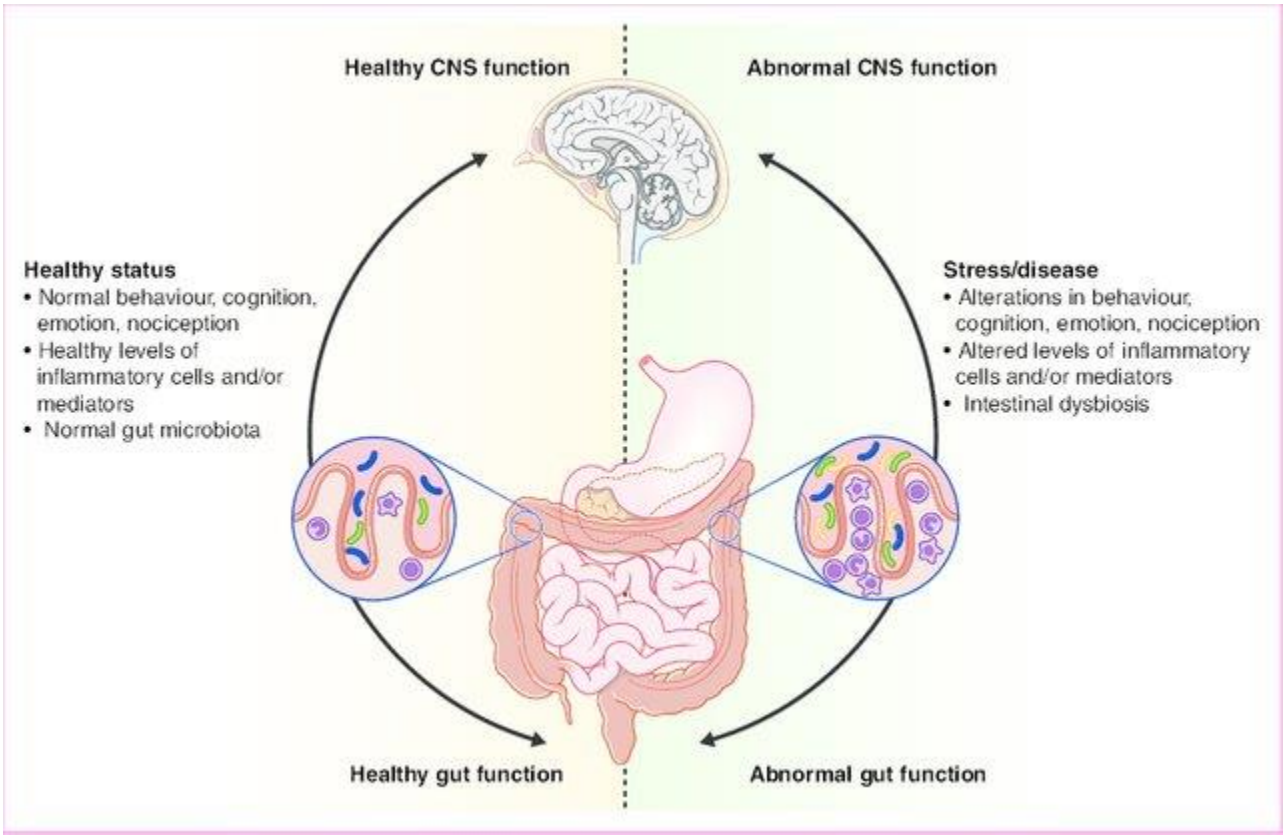

Figure 1.1: Gut-Brain axis[9]

1.3 Gut microbe and colonization resistance:

The gut microbiota harbors a wide range of complex and diverse microbes. This dense microbiota inhibits colonization and overgrowth of harmful microbes during healthy conditions. This phenomenon is known as colonization resistance [10]. Thus the gut microbiota plays a crucial role against invader pathogens. Four phyla are dominant in the human gut and include Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Each phylum harbors a high diversity of species and strains [11,12]. The bacteria such as *Roseburia*, *Ruminococcus*, or *Coprococcus* belonging to phylum Firmicutes, synthesize short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate [13,14]. These SCFAs exert a beneficial effect on the host energy metabolism [15,16]. SCFA, particularly butyrate, has immunomodulatory functions as well. These microbial SCFAs have a role in regulating intestinal epithelial cells. These cells play a significant role in maintaining mucosal immunity and intestinal homeostasis [17-20].

Bile salts are known to play a role by counterbalancing the pathogenesis of *C. difficile*. Bile salts are the conjugated forms of steroidal acids synthesized from cholesterol in the liver. Primary bile salts (cholate and chenodeoxycholate), after being synthesized in the liver [22], are released into the small intestine. Primary bile salts can induce germination of *C. difficile* spores. Later, the primary form of bile salt is converted into its secondary form (deoxycholate) [21] by the cecal microbiota. These secondary bile salts inhibit the vegetative growth of *C. difficile*. As a result, *C. difficile* cannot produce toxins, and a healthy environment is maintained in the gut [21].

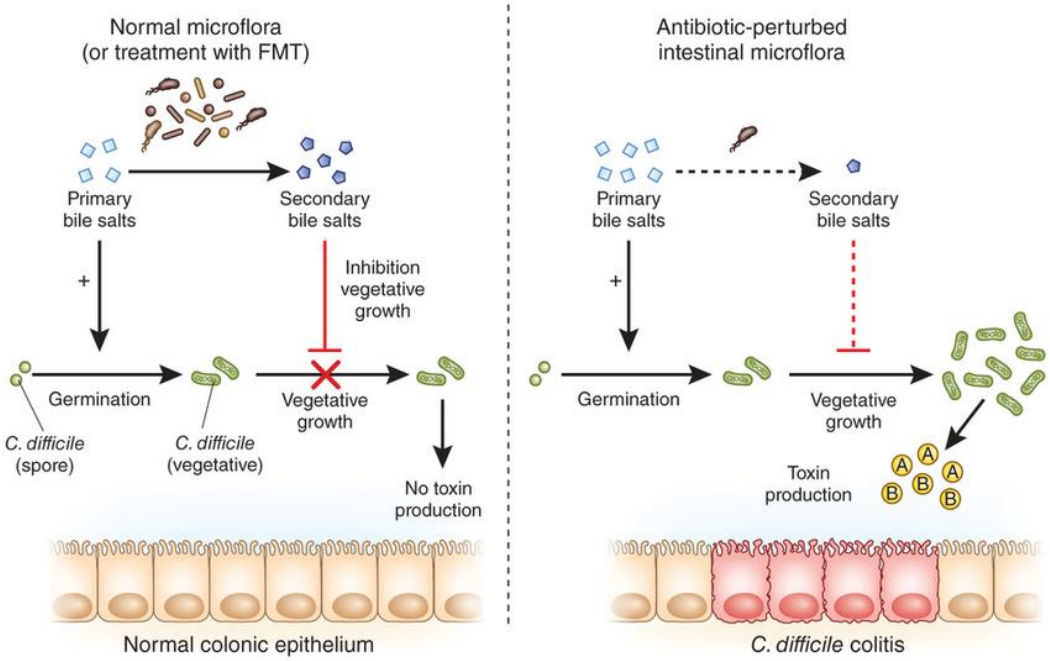

Figure 1.2: Mechanism of bile salts on CDI pathogenesis [21].

In this way, host-microbiota provides resistance to *C. difficile*. While we use antibiotics, it destroys the harmful bacteria and kills the beneficiary bacteria. While we lose these bacteria, *C. difficile* gets the chance to emerge and cause *C. difficile* infection (CDI). So, the chance of CDI is increased while someone stays in the hospital. This is called health care-associated CDI (HA-CDI).

1.4 Inter kingdom signaling:

In the human gut, a vast number of microorganisms co-exist. These microbes communicate through an extracellular signaling molecule called autoinducer Al-3 [32]. This process is known

as quorum sensing [33]. However, this process can also facilitate the interaction between microbes and their host, known as ‘inter-kingdom signaling” [34-36]. So, bacteria can maintain symbiotic or pathogenic relationships with their hosts. This phenomenon is also described using another terminology, ‘microbial endocrinology.’ Because sometimes, bacteria respond to chemical signals from the host and consequently release chemicals to modulate the host responses [37,38].

1.5 Two-component signal transduction system:

The hypothesis that microorganisms can actively respond to the host's neuroendocrine hormones, which can initiate growth and pathogenesis, got recognized over the last decade[39,40]. Most studies use catecholamines because of their sudden release and contact with the bacterial community during stress[41,42]. Catecholamines could directly facilitate the growth of gram-positive and gram-negative[43,44]. Subsequent studies were performed to demonstrate the role of catecholamines in toxin production [45], adhesions [46], and biofilm formation[47] [50]. It was found that bacteria can change their gene expression according to the environment. Bacteria can alter their gene expression through a mechanism known as a two-component system (TCS) [48,49]. Usually, two proteins are involved a sensing protein and a transcription factor. In this system, extracellular sensing domains (QseC) use kinases for transferring phosphate groups to DNA-binding molecules (QseB) [48,50]. As a result, the levels of gene expression are changed. QseC, two membrane-spanning domains that act as a histidine kinase, comprises a periplasmic sensor domain and a cytoplasmic kinase domain (figure 1.3). QseC is activated by the signal and regulates QseB. The QseB consists of a receiver domain and a helix turn helix (HTH) DNA binding domain.

However, the QseBC two-component system (TCS) is also involved in quorum sensing along with virulence gene expression [50]. In addition, QseBC is mainly distributed in the Enterobacteriaceae and Pasteurellaceae due to sequence similarity[50]. It was found that QseC works as an adrenergic receptor in Enterohemorrhagic *E. coli* (EHEC) [51] & *Aggregatibacter actinomycetemcomitans* [52]. In *Salmonella. enterica*, PreB, QseC paralog (87% sequence identity of QseC EHEC) works as an adrenergic receptor[53].

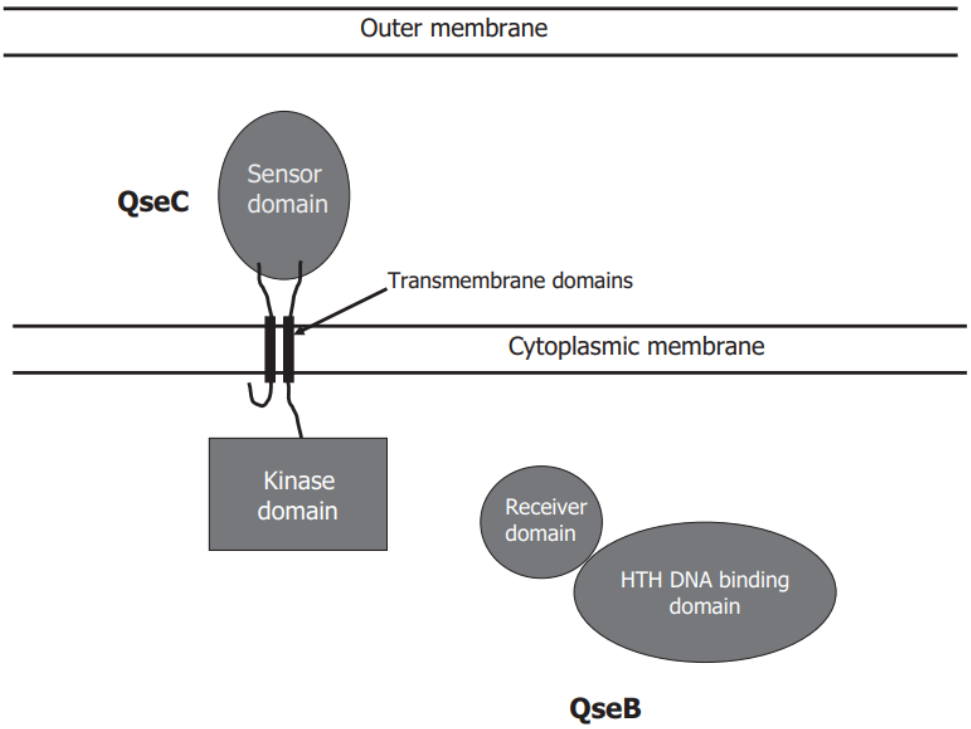

Figure 1.3: Schematic diagram of the QseBC two-component system[50].

1.6 Catecholamines facilitate bacterial iron acquisition:

Iron plays a crucial role in the growth and virulence of bacteria. Some bacteria can produce siderophores, while others use exogenous siderophores to obtain iron. Catecholamines behave as a siderophore for scavenging bound iron from the host[54] along with increasing autoinducer production. This phenomenon is observed in many infectious diseases, including periodontitis. Mucosal tissue fluid contains iron-binding proteins such as lactoferrin, transferrin, and glycoprotein, and these proteins build a bacteriostatic environment by sequestering free iron from the environment[55-58]. In *Porphyromonas gingivalis*, catecholamines promote the gene expression related to oxidative stress and virulence genes by increasing iron acquisition and strengthening the adaption of bacteria during oxygen stress conditions [59-61].

1.7 Norepinephrine (NE):

Norepinephrine (NE), a neurotransmitter, is synthesized from the adrenal medulla and postganglionic neurons of the sympathetic nervous system (SNS). After binding with the

adrenergic receptor, it takes the initiative to activate the receptors on targeted cells' surfaces. The serosal surface of the intestine is densely innervated by SNS [23]. In stress conditions, the body responds through the autonomic nervous system. The SNS, a part of the autonomic nervous system, triggers the 'flight or fight' response [24-26], and NE is released abundantly in the gut as part of the 'flight or fight response [27]. Although found to enhance the growth and virulence of other bacterial strains, including *Staphylococcus*, *Escherichia coli*, *Salmonella*, and *Vibrio* [28-31], the effect of NE was not tested in *C. difficile.* The current study investigates the response of *C. difficile* to norepinephrine in growth and virulence.

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CHAPTER 2. LITERATURE REVIEW

2.1 *Clostridioides difficile* infection (CDI):

C. difficile can cause life-threatening diarrhea and colitis, which is designated as *C. difficile* infection (CDI). When a person takes antibiotics, it can cause digestive system complications and deadly diarrhea [1]. One-third of the *C. difficile* infections (CDI) are acquired within the healthcare settings [2]; 24% of CDI occurs in hospitals, while 40% of cases begin in community healthcare centers [3]. According to the Antibiotic resistance & patient safety portal (2020) report, 3653 cases were reported from general acute care hospitals, while 113,451 cases were from hospitalized patients [4]. In addition, in acute care hospitals, the Standardized Infection Ratio (SIR) in general for CDI was 0.52, the highest in West Virginia (0.75) in 2020 [4]. There is a greater chance of recurrent infection in CDI patients.

2.2 Epidemiological study of CDI in the USA :

The Centers for Disease Control and Prevention (CDC) classifies infections with *C. difficile* as an urgent threat to the public because CDI may cause life-threatening diarrhea and pseudomembranous colitis, mainly in those who have had both recent hospitalization and antibiotics. According to the CDC's 2019 AR (antibiotic resistance) threat report, approximately half a million Americans are affected by *C. difficile* annually. Among them, 29,000 had deadly consequences after early diagnosis, and 15,000 of these deaths were driven by the CDI [1].

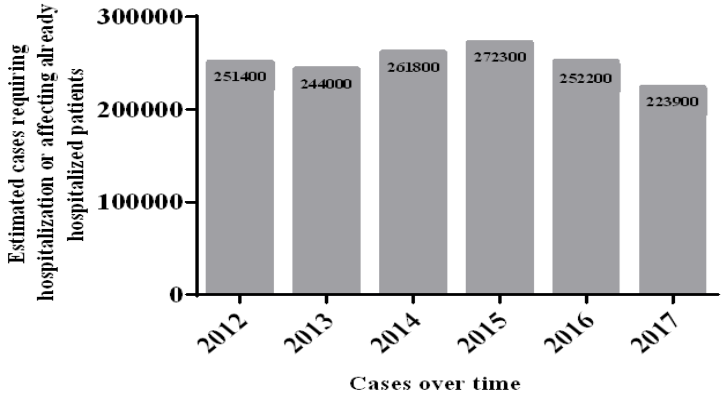

Figure 2.1: Cases of hospitalization due to CDI from 2012-2017 [1].

Approximately 83,000 of the patients with *C. difficile* had at least one recurrence. CDC estimated the total healthcare cost associated with CDI as nearly $1 billion in the US. CDC warned people that about 1 in 6 CDI patients could have recurrent CDI episodes within 2-8 weeks, and 1 in 11 CDI patients above 65 years old would die within 30 days [1].

2.3 An epidemiological study in other countries:

Though CDI is highly prevalent in Europe and North America, it is not uncommon in other continents. An estimated annual incidence of CDI is about 172,000 in Europe and 18,005 in England [5]. The highest incidence in Europe was reported in Poland; HA CDI at 6.18 and CA-CDI at 1.4 per 10,000 patient days [6]. In Australia, the average prevalence was estimated to be 4.0 per 10,000 patient days between 2011 and 2016. However, CDI among all patients with diarrhea was 14.8%, with a higher prevalence in East Asia (19.5%), compared with South Asia (10.5%) or the Middle East. Their estimated rate (5.3 CDI/ 10,000 patient-days) was close to Europe and North America. However, CDI-related mortality was 8.9% in Asia [7]. 2016 CDI incidence survey of France estimated CDI incidence in acute care was 3.6 cases per 10,000 patient days. There was a statistically significant increase in CDI incidence between 2010 and 2016 (+ 14% annually), and testing frequency was 47.4 per 10,000 PD [8]. In Canada, a total of 37,900 CDI cases were reported in 2012. 10,900 cases were reported as recurrent infections, of which 7,980 (21%) cases were relapses. The total expenses of society due to CDI were estimated as $281 million. Direct medical expenses were 12 million (4%), and $260 million (92%) were associated with hospital costs, while 10 million dollars was due to loss of productivity [9].

2.4 Risk factors:

CDI is a threat to public health globally. The major risk factors include antibiotic exposure, age (>65) of the patient, and length of hospital stay. Studies show that exposure to multiple antibiotics increases the risk of developing CDI. The gut microbial flora acts as a barrier for enteric pathogens via a colonization resistance mechanism in a healthy patient. Antibiotic usage disrupts the gut microbial community and promotes the colonization of opportunistic pathogens, including *C. difficile.* Metagenomic data revealed changes in the composition of fecal microbiomes of patients [10]. There was a decrease in symbionts and an increase in opportunistic pathogens in the fecal

microbiota. Similarly, patients with any underlying medical conditions such as cancer, HIV, or patients on immune-suppressive drugs are more prone to CDI [1].

2.5 Virulence factors of *C. difficile*:

In the late 1970s, *C. difficile* was identified as an etiological agent [11] responsible for antibiotic-associated diarrhea [12]. With the emergence of the hypervirulent strain, noticeable exposure of CDI incidence has been observed over the past decades in the UK, USA, Canada, and mainland Europe [13-15]. Several virulence factors are associated with CDI, including the two large clostridial toxins: *tcdA* and *tcdB*.

2.6 *C. difficile* toxins:

C. difficile produces two toxins, toxin A and toxin B, which cause an acute intestinal inflammatory response. These two mainly mediated the pathogenicity of *C. difficile*. These toxins primarily attack the epithelial cell of the intestine by disrupting cell structure and junction, causing apoptosis.

2.7 Toxin A*:*

Toxin A (ToxA) is the largest toxin, with a molecular mass of 308 kDa [19]. This toxin is encoded by the *tcdA* gene located along with *tcdB* on the pathogenicity locus (PaLoc; 19.6kb) [16,17]. PaLoc is found only in the toxicogenic strains, while non-toxigenic strains carry a 127 bp fragment in that position [18]. Though it is an enterotoxin [20], it also has some cytotoxic effects [21] on human intestinal epithelial cells (IECs) [20,21]. Primarily, ToxA binds and disrupts the tips of the intestinal villi. Thus, causing tissue injury via disruption in the epithelial barrier function of IECs and apoptosis, leading to pseudomembranous lesions. [22].

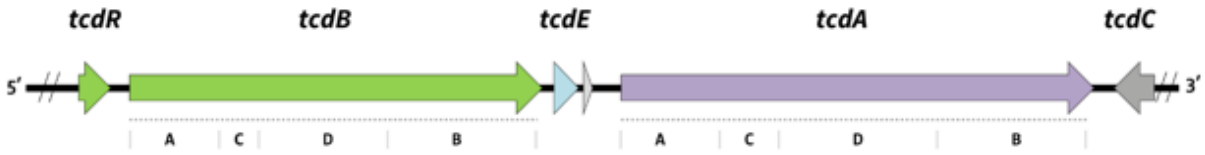

Figure 2.2: Image of PaLoc in the toxicogenic strain of *C. difficile* [23]

2.8 Toxin B:

Toxin B (ToxB) is another toxin among two major toxins, leading to fatal consequences by damaging intestinal epithelium and pseudomembranous colitis, leading to an acute inflammatory[24] response. This potent cytotoxin with a molecular weight of 270 kDa [25] damages the cytoskeleton and tight junction of the mammalian cell. However, the most severe cytotoxic effects are found in human cervical epithelial cells [26]. ToxB causes disturbances in the signal transduction mechanism, cell rounding, and apoptosis via the actin fiber of the targeted cell [27]. Usually, filamentous actin gives the cell a proper shape, integrity, and mechanical support. ToxB increases the quantity of cytosolic actin, a soluble form of the actin filament. Consequently, the level of filamentous actin is decreased, and cells lose their integrity [28].

2.9 Other virulence factors:

2.10 Flagellin gene *fliC*:

Flagellin gene *fliC* is one of the virulence factors. The molecular mass of *fliC* protein is 39kDa [29]. *C. difficile* needs to adhere and colonize to interact with its' targeted cell. The *fliC* protein has the adhesive property [30]. If unable to attach and colonize into the targeted epithelial cells, a nonspecific defense mechanism quickly eliminates these bacteria [31,22].

2.11 *Spo0A*:

C. difficile forms spores during adverse conditions. These reproductive endospores are produced from vegetative cells, which are metabolically dormant [32]. However, these spores are highly infective form and can also survive outside the host even though this pathogen is oxygen-sensitive [33]. These spores are resistant to commonly used disinfectants and, as a result, become a potential transmission reservoir [34-36]. Sin locus is located in *C. difficile* genome as a central link that connects the gene regulatory networks of sporulation. *Spo0A*, the master regulator of sporulation, controls this *sin* locus expression by binding its upstream region [38] and initiating sporulation [32]. Moreover, the *sin* locus has two pleiotropic regulators and is labeled as *sinR* (112aa) and *sinR'* (105aa) in the operon, respectively [37].

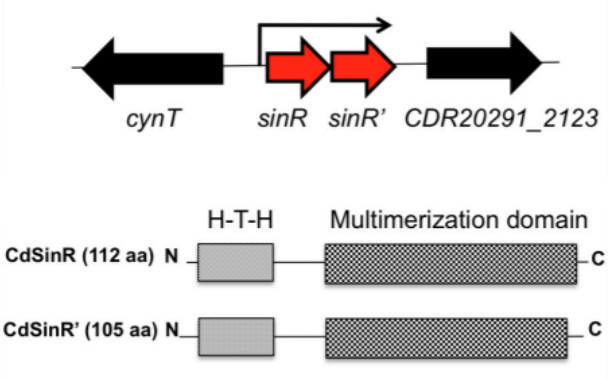

Figure 2.3: Sin Locus of *C. difficile* R 20291[38].

2.12 Pilin genes:

C. difficile produces multiple toxins responsible for causing chronic diarrhea and pseudomembranous colitis. This disease condition is generated while bacteria bind their host cell and secrete toxins through a type IV secretion system (T4SS) [39,40]. In this secretion system, bacteria create an extracellular structure with an injector to transfer toxins [41], leading to infectious diseases. This extracellular structure consists of different pilin proteins. The major and minor subunits of T4SS are PilA1 [43,45], and PilJ, respectively [42,44]. PilA3, PilA5, and pilA6 are other pilin proteins.

2.13 Pathophysiology:

C. difficile is a nosocomial pathogen, and the incidence of *C. difficile* infection (CDI) has been increasing since 2000 [45]. It is frequently diagnosed in older adults and hospitalized patients [4]. However, CDI symptoms can appear within 48 hours after hospitalization or 4 weeks after discharge. Even without antibiotic therapy or hospitalization, a considerable percentage of the world population suffers from CDI, which is noticeable [45]. This group is classified as community-acquired CDI. Sometimes symptoms are appeared 12 weeks later discharged from the hospital. [46]**.**

2.14 Route of transmission:

C. difficile spores can survive for long periods in the environment. Once entered into the susceptible host, these spores germinate to a vegetative form that could initiate *C. difficile infection*

under suitable conditions. *C. difficile* is spread via the fecal-oral route [59]. The organism is ingested either as the vegetative or as spores form[59,60]. If resistant spores are ingested, they can survive and pass through the stomach and ultimately colonize the colon, creating further complications [61].

2.15 Clinical manifestations of CDI:

Symptoms of CDI are diverse. Some people carry CDI in their gut but never become sick. These individuals may spread infections as asymptomatic carriers. However, infection is not the only prerequisite for developing severe symptoms. Disturbance of the intestinal flora initiates symptom development. The most common symptoms are watery diarrhea and abdominal cramping. People may develop several symptoms in severe cases, such as fever, dehydration, loss of appetite, inflamed colon, etc.

2.16 Carrier stage:

Carriers are those individuals who serve as a reservoir by shedding *C. difficile* in stools but surprisingly do not have diarrhea. This asymptomatic carrier is a prospective source for transmission of epidemic and nonepidemic strains [45]. Several statistical analyses showed that the carrier frequency in healthy adults is nearly 3%, but in hospitalized patients, it is 20-30%, and in patients with extended hospital stays, it is 50%. Though asymptomatic individuals served as potential reservoirs in the hospital environment, the patients having symptoms of diarrhea spread more spores [47].

2.17 *C. difficile*-Associated Diarrhea (CDAD):

C. difficile is the cause of diarrhea in the hospital environment in patients during their course of antibiotic treatment or at the end of their antibiotic course. Even in long-term care facilities (LTCFs), antibiotics are frequently used. As a result, older adults of LTCFs suffer from *C. difficile* colonization and diarrhea [48]. This type is known as antibiotic-associated diarrhea, and approximately 25–30% of all cases are usually found due to this reason [47]. According to the Society for Health care facilities of America (SHFA), this bacterium has been identified as the

most common cause of non-epidemic acute diarrheal illness and outbreaks of CDAD in the nursing home and LTCFs [48].

However, diarrhea starts with severe abdominal pain and cramping. Sometimes 10-15 times bowl movements are recorded in patients leading to electrolyte imbalance. Even CDAD can cause death in a patient suffering from serious illness, especially after surgery [45]. Interestingly, ToxA is identified in the patient's stool suffering from severe CDAD [49].

2.18 *C. difficile*-Associated Colitis (CDAC)

C. difficile can cause colitis without pseudomembrane formation. In this case, patients suffer from dehydration, nausea, watery diarrhea, fever, and abdominal cramping. Trace amounts of blood are noticed in severe cases [50].

2.19 Pseudomembranous Colitis

Pseudomembranous colitis was first described in 1983 [51]. Clinical signs include watery diarrhea, severe dehydration, mucus, and hypoalbuminemia (less than 30mg/ml) [45]. Patients with pseudomembranous colitis have yellowish plaques in their colorectal mucosal area and ileum (Fig 2.4).

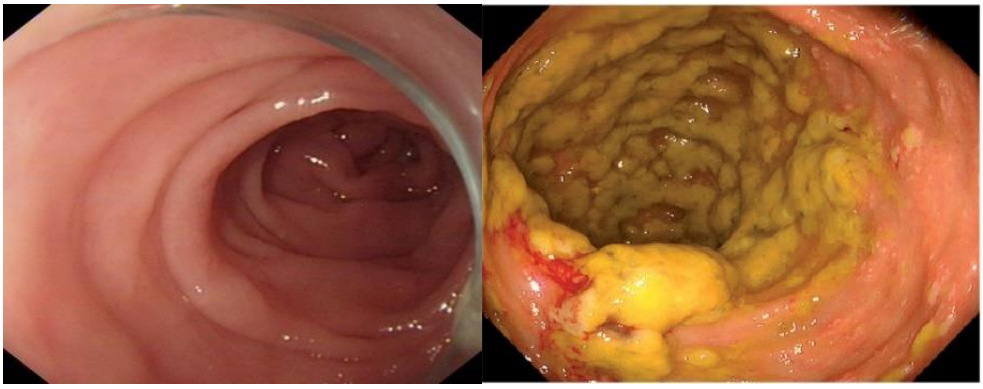

Figure 2.4: Comparison between a normal colon and hemorrhagic sigmoidal colon [58]

2.20 Fulminant Colitis

Fulminant colitis is a rare and most severe form of CDI caused by hypervirulent strains of *C. difficile* [44,45]. Approximately 3-8% of CDI patients have this acute colitis. But diagnosis is difficult in 20% of patients due to the absence of diarrhea. The symptoms include perforation in the ileum, megacolon, leukocytosis, and other complexities of colitis, leading to the death of these patients [52].

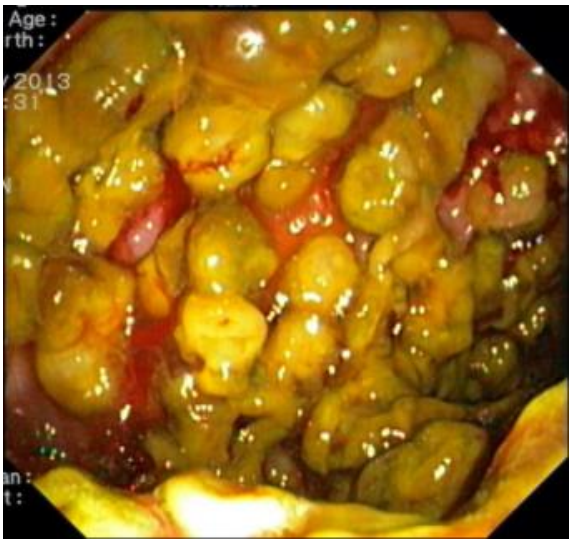

Figure 2.5: Image of fulminant colitis with multiple polypoid lesions. [57]

2.21 Extracolonic Infections

Usually, *C. difficile* is recognized as a gastrointestinal pathogen. But recently, it has been found that this pathogen can cause empyema, intraabdominal abscess, appendicitis, visceral abscess, reactive arthritis, and osteomyelitis [54,55,59].

2.22 Recurrent CDI

The recurrent rate of CDI is high, and it is one of the greatest challenges [45,46] to control. Even after successful treatment of CDI, recurrent CDI often occurs. New virulent strains are responsible for causing 33%–75% of cases of recurrent CDI. Though the exact reason behind the recurrent CDI has not been recognized, the interruption of the normal microbiota and defective and impaired immune response against *C. difficile* could be responsible for developing recurrent CDI [53].

2.23 CDI treatment:

Risk factors for CDI infection include old age, long-term treatment with antibiotics, and extended hospitalized stays. Based on the current guidelines, the most common drugs to treat CDI are Metronidazole, Vancomycin, and Fidaxomicin.

Depending on the severity of CDI, one of these antibiotics are prescribed.

2.24 Metronidazole:

Metronidazole, a common antibiotic used as a first-line treatment for mild-to-moderate CDI, belongs to the nitroimidazole class of antibiotics [69]. Metronidazole enters the bacterial cell via passive diffusion. After entry, the antibiotic is reduced by pyruvate: ferredoxin oxidoreductase system of the bacterial cell and is converted into an active form following reduction of the nitro group to nitro radicals. This reduction leads to the production of toxic metabolites in the cell environment that interacts with bacterial DNA and cause the breakage of DNA strands.

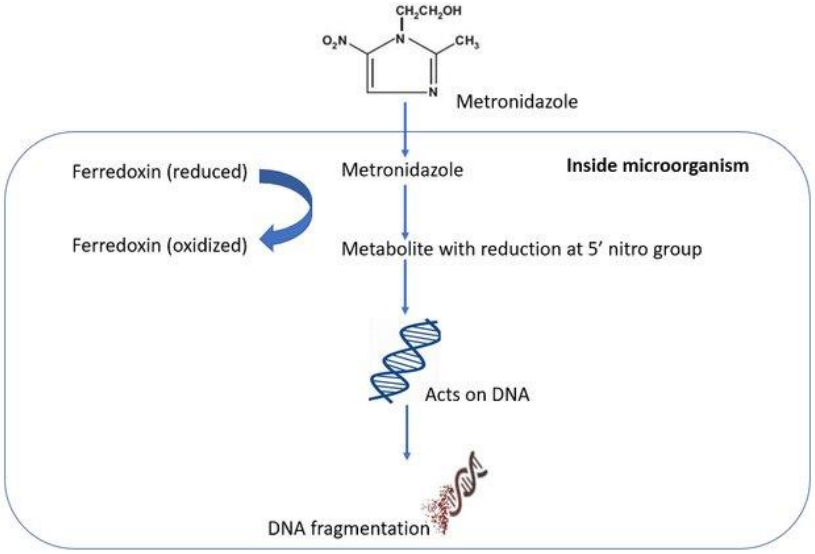

Figure 2.6: Mechanism of action of Metronidazole [68]

2.25 Fidaxomicin:

Fidaxomicin is a first-in-class macrocyclic antibacterial agent [62]. It shows its antibacterial effects by inhibiting bacterial RNA polymerase at the earlier step [63] of transcription initiation [64,65]. In the transcription process, RNA polymerase binds with the DNA template and opens the DNA

strand for further mRNA formation. Fidaxomicin hampers mRNA formation by binding with the complex and inhibiting the σ subunit of the complex [65,66].

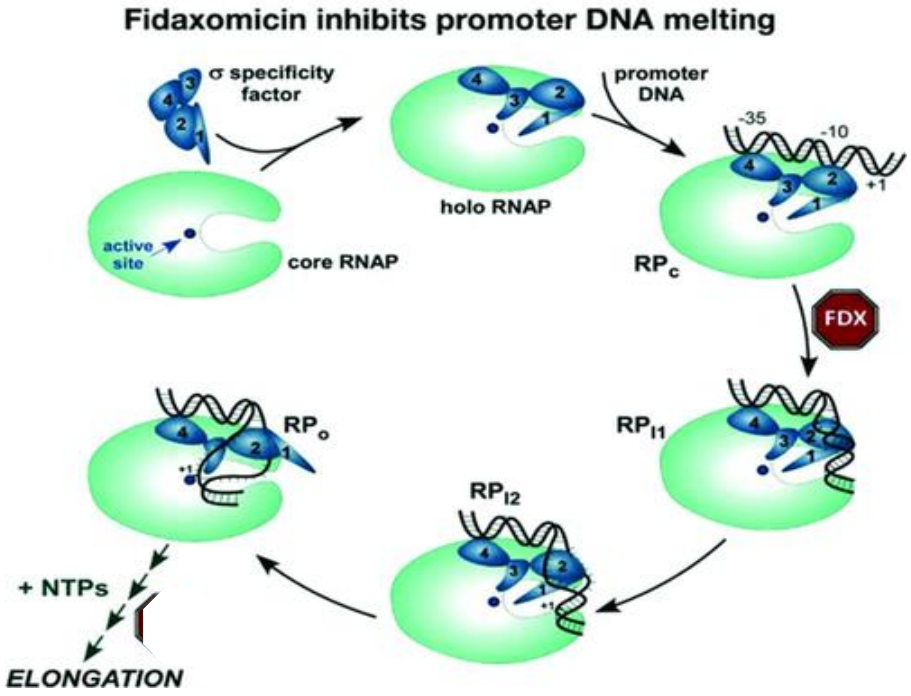

Figure 2.7: Mechanism of action of Fidaxomicin [67]

2.26 Response of *C. difficile* to low iron conditions:

Pathogenic bacteria need to survive the host environment to establish an infection. Iron is an essential nutrient required for the growth of bacterial and host cells, and hence, the is competition between host and bacterial cells iron. *C. difficile* has developed ways to overcome low iron bioavailbility in the environment through an iron regulator, ferric uptake regulator (Fur) protein, found in *C.difficile* [70]. Using ferric uptake regulator, *C. difficile* expresses different pathways for iron uptake, such as exhibiting binding sites for Fur [71] and replacing the pathways that require iron-containing proteins with alternative iron-deficient mechanisms.

To protect from host immune responses or antiacterial medication in iron deficient conditions, *C. difficile* changes the cell wall composition of its own, including up-regulation of virulence-associated factors, such as flagella-associated genes [71,72].

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CHAPTER 3. METHODS

3.1 Background

Clostridioides difficile (formerly known as *Clostridium difficile*), is a Gram-positive, anaerobic, spore-forming bacterium that causes antibiotic-associated diarrhea in humans. According to the 2018 Emerging Infections Program (EIP) report of CDC, 15,591 cases of CDI were reported in 35 counties in 10 states [1]. Virulent strains of *C. difficile* generally produce two toxins (Toxin A and Toxin B) that have been identified as major virulence factors [2]. Along with toxin production, several virulence factors of *C. difficile* include spore formation, biofilm formation, surface adhesins, and other surface structures (e.g., pilus and flagellum), and motility are responsible for *C. difficile* associated infection [3]. Norepinephrine (NE), the stress-associated neuroendocrine hormone, modulates the bacterial behavior of several Gram-positive and Gram-negative bacterial species, including *Staphylococcus, Escherichia coli, Salmonella* and *Vibrio* [4-7]. The current study investigates the response of *C. difficile* to norepinephrine in growth and virulence.

3.2 Materials and methods

3.2.1 Bacterial strains, media, and growth condition:

Studies were carried out with *C. difficile* strains detailed in Table 3.1. Bacterial strains were obtained from the American Type Culture Collection (ATCC) and Biodefense and Emerging Infections (BEI) Research Resources repositories. All strains maintenance was performed routinely by growing the cells in an anaerobic BHIS (brain heart infusion-supplemented with yeast extract and L-cysteine) medium in an anaerobic chamber (95 % N_2/5%H_2) regularly at 37°C, as detailed in Table 3.2. For solid medium, agar was added to a final concentration of 2.0% (wt/vol).

Table 3.1: *C. difficile* strains used in this study

	Strains	Alternate designation/ Catalog No.	Year of isolation	Sources	Description
1	P8	NR-32888	2001	Isolated from fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxicogenic strain
2	P13	NR-32891	2005	Isolated from the fecal material of a human patient with *C. difficile* infection in Western Pennsylvania, USA	Toxicogenic strain
3	Isolate 20100502	NR-49277	2010	Obtained from the stool of an older adult male patient with a community-associated *C. difficile* infection in Colorado, USA	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3], and *tcd*[4]*C* of the PaLoc[5] operon, as well as the CDT[6]
4	Isolate 20120187	NR-49290	2011	Obtained from the stool of an elderly male patient with a healthcare-associated *C. difficile* infection in Tennessee, USA	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3], and *tcd*[4]*C* of the PaLoc[5] operon, as well as the CDT[6]
5	Isolate 20120016	NR-49282	2011	obtained from the stool of a pediatric female patient with a community-associated (CA) *C. difficile* infection in New York, USA	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3] , and *tcd*[4]*C* of the PaLoc[5] operon, as well as the CDT[6]
6	ATCC 43255	VPI 10463		Human abdominal wound	PCR ribotype 087, *tcdA*[2], *tcdB*[3] toxinotype 0

NAP 1= North American pulsed-field gel electrophoresis type 1.

tcdA= *C. difficile* toxin A gene.

tcdB= *C. difficile* toxin B gene.

tcdC= Anti-sigma factor gene.

PaLoc= Pathogenicity locus.

CDT= *C. difficile* binary toxin.

Table 3.2: Composition of Brain heart infusion supplemented (BHIS) media.

Component	Amount(per liter)
Brain heart infusion broth (dehydrated)	37.0 g
Yeast extract	5.0 g
Distilled water	1.0 L
Resazurin solution (25 mg/100 ml distilled water)	4.0 ml
Boil, cool, add	
L-Cysteine . HCl	0.5 g
Hemin Solution	10.0 ml
Vitamin K1	0.2 ml
Hemin Solution preparation:	
Hemin	50.0 mg
1N NaOH	1.0 ml
Distilled water (for making100.0 ml)	100.0 ml
Vitamin K1 Solution preparation:	
Vitamin K1	0.15 ml
95% Ethanol	30.0 ml

3.2.2 Growth curves and toxin production

For growth curve formation, the overnight culture of *C. difficile* strains was diluted 1:100 ratio in freshly prepared, pre-reduced BHIS media. Two concentrations of Norepinephrine (NE) 5 & 50µM (L-(-) NE bitartrate hydrate (Cayman Chemical, CAS registry No: 108341-18-0) were used to study the effect on bacterial growth. In contrast, an equal volume of DMSO was used to treat bacteria as an experimental control. Each strain was grown in 3 biological replicates. Bacterial growth at 37 °C was determined by the optical density (O.D.) at 600 nm. The OD was recorded every hour for 8 hours. After 8 hours, the bacterial culture was transferred to an Eppendorf tube, centrifuged for 5 min at 10,000 rpm, and the supernatant was transferred to a fresh Eppendorf tube and stored at -80^{o}C for toxin analysis.

For RNA isolation, RNA later was added to the bacterial pellet and kept at 4^{o}C overnight. The next day, the tubes were centrifuged (5 min at 8000 rpm), RNA later was discarded, and the bacterial pellets were stored at -80^{0}C until further analysis.

3.2.3 RNA Extraction and assessment of RNA quality and quantity

Total RNA was isolated from *C. difficile* strains grown in a BHIS medium after 8 hours of growth. RNA was extracted using an RNeasy mini kit (Qiagen, LOT56903404 & REF 74104) and treated with RNase-free DNase set (Qiagen, LOT 56904522 & REF 79254) as recommended by the manufacturer. Then performed total RNA MiniElute Cleanup with DNase digestion using Qiagen RNeasy protocol. Finally, RNA concentration was measured by Epoch Microplate Spectrophotometer using GEN 5 software.

3.2.4 No Reverse Transcriptase (RT) control test

For monitoring genomic DNA contamination, no reverse transcriptase control (–RT or no RT) reaction was performed. The procedure for cDNA synthesis was followed (as mentioned below), except an equal volume of water was used instead of Quantiscript Reverse Transcriptase. Finally, qPCR (elaborated 3.2.6) was performed. Any samples with the cycle threshold (CT) curve after the threshold level (ΔRn 0.1, ~30 cycles) was considered as "contamination-free DNA" and was used for further qPCR reaction.

3.2.5 Reverse Transcriptase PCR and cDNA synthesis

The cDNAs were synthesized using QuantiTect Reverse Transcription kit (Qiagen, LOT 169033079 & REF 205311) according to the manufacturer's protocol. gDNA wipeout buffer was used to remove the genomic DNA from RNA (provided with the kit). 1 µg RNA was used in the reaction mixture.

Table 3.3. Genomic DNA elimination reaction components

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA, 1 µg	Variable
RNase-free water	Variable
Total reaction volume	14 µl

The total reaction volume was incubated for 2 min at 42°C, then placed immediately on ice. The reverse transcription master mix preparation is mentioned in Table 3. 4. All these reaction mixtures were prepared on ice.

Table 3.4: Reverse-transcription reaction components

Component	Volume/reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase (contains RNase inhibitor)	1µl
Quantiscript RT Buffer, 5x (contains Mg2+ and dNTPs)	4µl
RT Primer Mix	1µl
Template RNA Entire genomic DNA elimination reaction (Table 3)	14µl
Total reaction volume	20µl

After adding the template RNA, the reaction mixture was incubated at 42°C for 15 min. Later, the reaction mixture was incubated at 95°C for 3 min for inactivating Quantiscript Reverse Transcriptase. At the end of the reaction, the total 20µl volume reaction mixture was stored at –20°C for qPCR analysis.

3.2.6 Quantitative real-time PCR (qPCR) analysis:

Power up ™ SYBR ™ Green Master Mix (applied biosystems by Thermo Fisher Scientific, LOT 01143681 & REF A25742) was used. The qPCR reaction mix was set up as mentioned in Table 3.5, and the standard cycle mode was performed as mentioned in Table 3.6. This study used "Power

up ™ SYBR ™ Green Master Mix" (Thermo Fisher Scientific, LOT 01143681 & REF A25742. In the master mix, 10ng cDNA (cDNA concentration 5ng/µl) and 500nM of each primer was added, making a total volume of 10 µl qPCR reaction mixture.

Table 3.5: qPCR reaction component

Component	Volume
Power up ™ SYBR ™ Green Master Mix (2x)	5µl
Forward primers	0.5µl
Reverse primers	0.5µl
cDNA template	2µl
Nuclease free water	2µl
Total reaction volume	10µl

Table 3.6: Standard cycling mode (primer Tm<60^0C)

Step	Temperature	Duration	Cycles
Activation	50^0C	2 minutes	Hold
Dual-Lock DNA polymerase	95^0C	2 minutes	Hold
Denature	95^0C	15 seconds	40
Anneal	55-60^0C	15 seconds	
Extend	72^0C	1 minute	

In the annealing step, the temperature was set up according to the melting point of the primers (Table 3.7). All reactions were set up in a 96 well PCR plate (Fisher brand, Cat No. 14230232).

Table 3.7: Primer sequence along with melting temperature

	Primer		Sequence	Tm
1	***tcdA***	**Forward**	**5′-GGG GAT CGAGAC ACA CAG T-3′**	**58**
2	***tcdA***	**Reverse**	**5′-GCT CCA GTT TCC CAC CAA AA-3′**	**58**
3	***tcdB***	**Forward**	**5′-GGA GAA TGG AAG GTG GTT CA-3′**	**57**
4	***tcdB***	**Reverse**	**5′-CTG GTG TCC ATC CTG TTT CC-3′**	**58**
5	***flagellin***	**Forward**	**5′-TTG GAA CAA ATG TTG CAG GA-3′**	**56**
6	***flagellin***	**Reverse**	**5′-TTG TGC CCC TAA TTT TGC TC-3′**	**57**
7	***pilA1***	**Forward**	**5′-TGG CAG TTC CAG CTT TAT TTA GTA AT-3′**	**57**
8	***pilA1***	**Reverse**	**5′-AAG ATA ATG CTG CAC TCT TAA TTG AA-3′**	**55**
9	***pilA3***	**Forward**	**5′-ACT TTC TTA AAC TAT GTC CAA AGT GA-3′**	**58**
10	***pilA3***	**Reverse**	**5′-TAC GAC AGT TCC ATC TTC TAG TTT ATC T-3′**	**57**
11	***pilA5***	**Forward**	**5′-CTC TAA GTG TAG AGA CAT TGA AGG A-3′**	**55**
12	***pilA5***	**Reverse**	**5′-TCC GCT ATC ATT TAT GAC AAG TTC G-3′**	**57**
13	***RpoC***	**Forward**	**5′-CAT TCA GGA GCC AGA GGT TC-3′**	**59**
14	***RpoC***	**Reverse**	**5′-GAC CTT TTC TGG CAC CAT GT-3′**	**59**

In each sample, the relative expression for a gene was calculated relatively to the *RpoC* gene. The assay was performed on a QuantStudio 3.0 real-time PCR instrument (Applied Biosystems) using the QuantStudio 3.0 software (Applied Biosystems). The relative change in virulence gene expression was recorded as a fold change of normalized target concentrations ($2^{-\Delta\Delta Ct}$).

3.2.7 Statistical analysis

All data analysis was performed with GraphPad Prism 8.0.2 (GraphPad Software Inc., USA). Data from the growth curve analysis were expressed as mean $\pm$ SD. In the case of the growth curve, differences between treated and untreated groups were explored by two-way ANOVA followed by Tukey's multiple comparison test. For virulence gene expression analysis, data were represented in Box and Whisker Plot and medians (interquartile ranges [IQR]) and were compared using one-sample t-test. A two-sided P value of <0.05 was considered statistically significant.

3.3 Motility assay:

For motility assay, following *C. difficile* strains were used.

Table 3.8: *C. difficile* strains used in motility assay

	Strains	Alternate designation/Catalog No.	Year of isolation	Sources	Description
1	Isolate 20120187	NR-49290	2011	Obtained from the stool of an elderly male patient with healthcare-associated *C. difficile* infection in Tennessee, USA	PCR ribotype 019, NAP1[1], contains *tcdA*[2], *tcdB*[3], and *tcd*[4]*C* of the PaLoc[5] operon, as well as the CDT[6]
2	ATCC BAA 1870	4118	2011	Clinically isolated from Maine , USA	PCR ribotype 027, NAP1[1], contains *tcdA*[2], *tcdB*[3] toxinotype IIIb
3	ATCC 43255	VPI 10463		Human abdominal wound	PCR ribotype 087, *tcdA*[2], *tcdB*[3] toxinotype 0

The overnight culture of *C. difficile* strains was diluted 1:100 ratio in fresh pre-reduced BHI media. Cultures of *C. difficile* were grown to mid-exponential phase (6-8hour) in BHI broth under the anaerobic condition at 37°C. For the assay, a semisolid agar culture tube was made of a BHI broth medium (37 gm/L) containing 0.4% agar with 50µM NE. The control tube received an equal volume of DMSO. Culture tubes were prepared by adding 5ml of BHI agar media into each tube and let to set for 2 hours. Then the tubes were transferred into the anaerobic hood and allowed to be pre-reduced for 6-8hour before inoculating *C. difficile* strains. Motility was measured by stabbing the tubes with 5µl of a strain followed by incubation at 37^0C for 8 hours.

3.4 Antibacterial activity of the anticlostridial drug:

Minimum inhibitory concentrations (MIC) of Metronidazole and Fidaxomicin antibiotics in the presence of norepinephrine were investigated in two strains of *C. difficile* NR 49290 and VPI 10463. MICs of these strains to these antibiotics were determined by the broth macrodilution method (Clinical and Laboratory Standards Institute , 1997).

3.5 References

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CHAPTER 4. RESULTS

4.1 Background:

Clostridioides difficile is an anaerobic bacterium that is a common inhabitant in our gut. When the levels of gut bacteria become imbalanced for taking antibiotics, this benign resident could multiply and cause life-threatening diarrhea. According to the 2019 Antibiotic Resistance Threats Report (AR Threats Report) of the CDC, infection due to antibiotic-resistant bacteria and fungi exceeds 2.8 million while it causes 35,000 deaths in the USA per year. [1]. Though *C difficile* is not typically resistant but can cause deadly diarrhea while taking an antibiotic and makes the situation critical and creating extra pressure along with antibiotic-resistant bacteria.

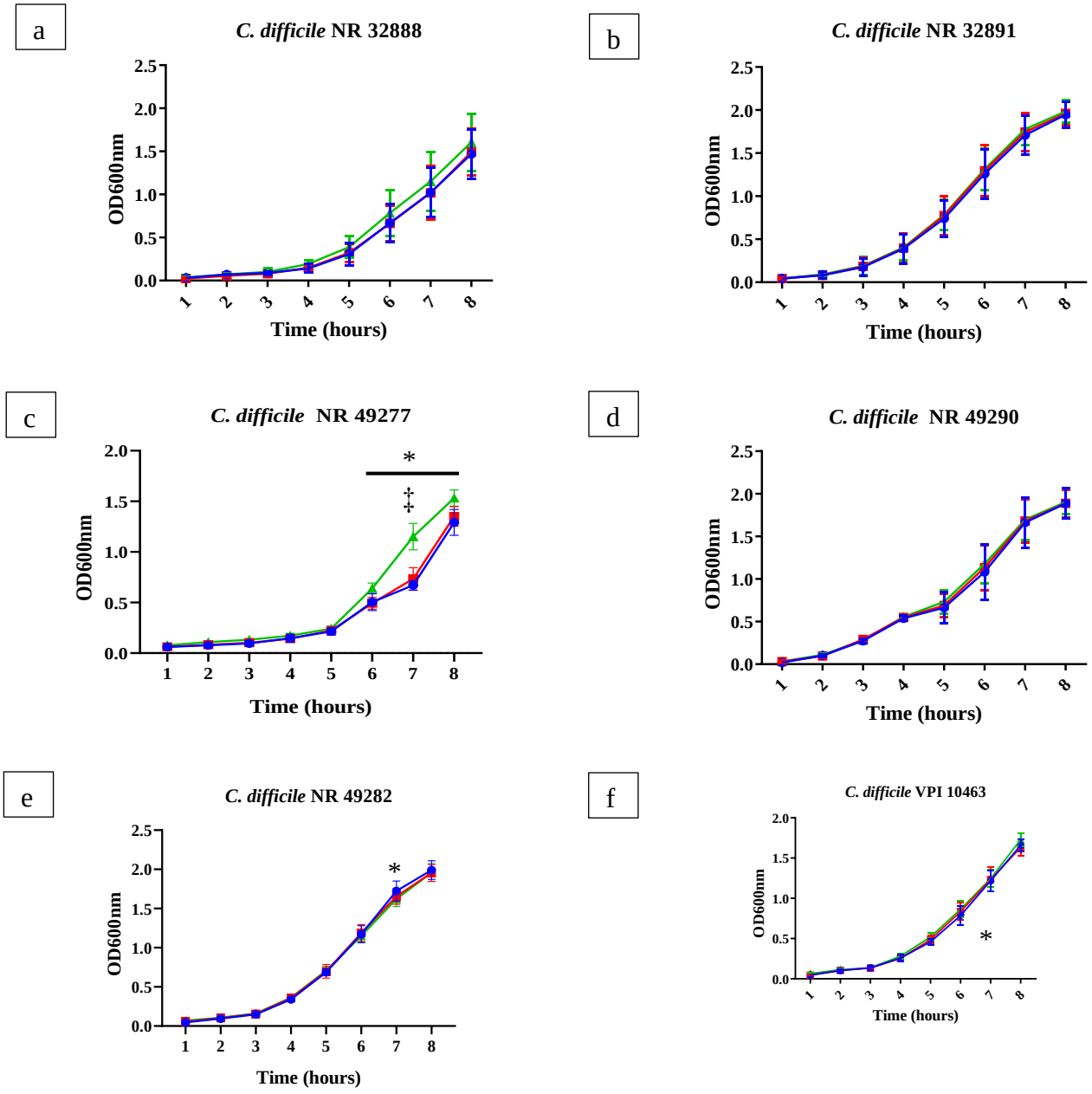
C. difficile-related severity was not identified earlier. But at the beginning of the millennium, a significant change in CDI severity, an increase in the case number, hospitalization, and recurrent infection was notified. Not only did the CDC declare CDI as an urgent threat but also Europe, Asia, the Middle East, and Central and South America recognized it's a concerning issue in several health reports. CDI could also occur in young and healthy individuals with no recent history of hospitalization or antibiotics exposure. Young individuals falling in the low-risk group could also experience CDI-associated diarrhea due to extended hospital stays and antibiotic treatment. [2-5]. This drastic change in behavior of *C. difficile* in the gut is attributed to the emergence of the hypervirulent strains. These hypervirulent strains expressed several *C. difficile* virulence factors compared to the regular strains. However, there was no difference in growth rates compared to regular strains [6]. *C. difficile* has two toxins (*tcdA* and *tcdB*) which are released more by hypervirulent strains in all their growth phases and, as a result, cause severe infections, longer hospital stays, and death. Furthermore, this hypervirulent strain also produces another toxin (*C. difficile* binary toxin, CDT) and the previous two, which play a critical role in the recurrence of CDI. Besides, a hypervirulent strain can produce more spores than a non-hypervirulent one [7,8], which increases recurrence rates and more outbreaks of both hospitals- and community-acquired infections [9-11]. In the GI tract, spores can persist as dormant and later germinate at the end of anticlostridial therapy, leading to recurrent infection. As a result, hypervirulent strains are considered one of the major risk factors for recurrent CDI [6,12].

Factors such as binary toxins, hypervirulent strains, dormant spores, anticlostridial therapy, and prolonged hospitalization could cause stress conditions in a patient. So, in this study, we tried to investigate *C. difficile* response to NE, one of the stress-associated neuroendocrine hormones, under such stress conditions.

4.2 Effect of NE on the *C difficile* growth:

The effect of NE on the growth of *C. difficile* was investigated *in vitro* using NE 5 & 50μM concentrations. Optical density was measured every hour until 8 hours to measure the bacterial growth in different concentrations of NE. The growth curves for all the 6 strains tested are shown below (Figure 4.1).

There was no difference in cell density for Strains NR 32888 (fig 4.1 a) & NR 32891 (fig 4.1 b) showed no difference in their cell density in the presence or absence of NE at both concentrations compared to the control. For strain 49277 (fig4.1 c), the addition of 50 μM NE significantly stimulated the growth after 6 hours and continued till 8 hours ($p<0.0001$). But no increased growth was observed in strain NR 49290 (fig 4.1 d). For strain NR 49282 (fig 4.1 e), after 7 hours ($p <0.01$), the growth was decreased in the presence of 50 μM NE when compared to the control. In strain VPI 10463 (fig 4.1 f), higher growth was observed at 6 hours (treated with NE 50 μM, $p<0.01$).



*=significant difference using 2-way ANOVA

‡=significant difference using Tukey's multiple comparison test

Figure 4.1: Effect of different concentrations of the NE (5&50 µM) on the growth of *C. difficile* in BHI medium supplemented with yeast extract & L-cysteine. For some points, the error bars showing SD of 3 biological replicates. The control was supplemented with an equal dosage of DMSO as 0 µM NE.

There was no difference in cell density between control & 5μM NE treated media for all strains tested.

4.3 Effect of NE on *C. difficile* toxin & flagellin genes:

The effect of NE on the expression of two toxin genes and one flagellin gene was investigated in *C. difficle* strains.

For toxins gene expression (*tcdA* & *tcdB)*, highest upregulation was observed (~8.3fold-P<0.05, ~8.8 fold-P<0.01 & &~14 fold-P<0.01) in strain NR 49290 after 8 hours incubation with 50 μM NE (figure 4.2 d) .In contrast, the addition of NE (5 & 50 μM) resulted in markedly downregulated expression of both toxin genes in NR 49282 (figure 4.2 e). In NR 32888 strain, both toxin genes were down-regulated at lower NE concentration (5μM), whereas upregulation was observed at higher NE concentration (50μM) (figure 4.2a). In NR 32891 strain, toxic gene *tcdA* was down-regulated while *tcdB gene* was upregulated at both concentrations (figure 4.2b)

Flagellin gene expression in all strains showed varied expression at different concentrations of NE. An upregulation was observed in strains NR 49277 (~ 8.5 fold-P<0.05), NR 49290 (~14 fold-P<0.01), VPI 10463 (~8.8 fold- P<0.01) at higher NE concentration (50μM) (figure 4.2 c, d, f) . In NR 49282, *flagellin* genes were downregulated at both concentrations of NE 5 & 50 μM (figure 4.2 e). In NR 32888 strain, also downregulation of the flagellin gene was observed at both concentrations of NE (figure 4.2 f).

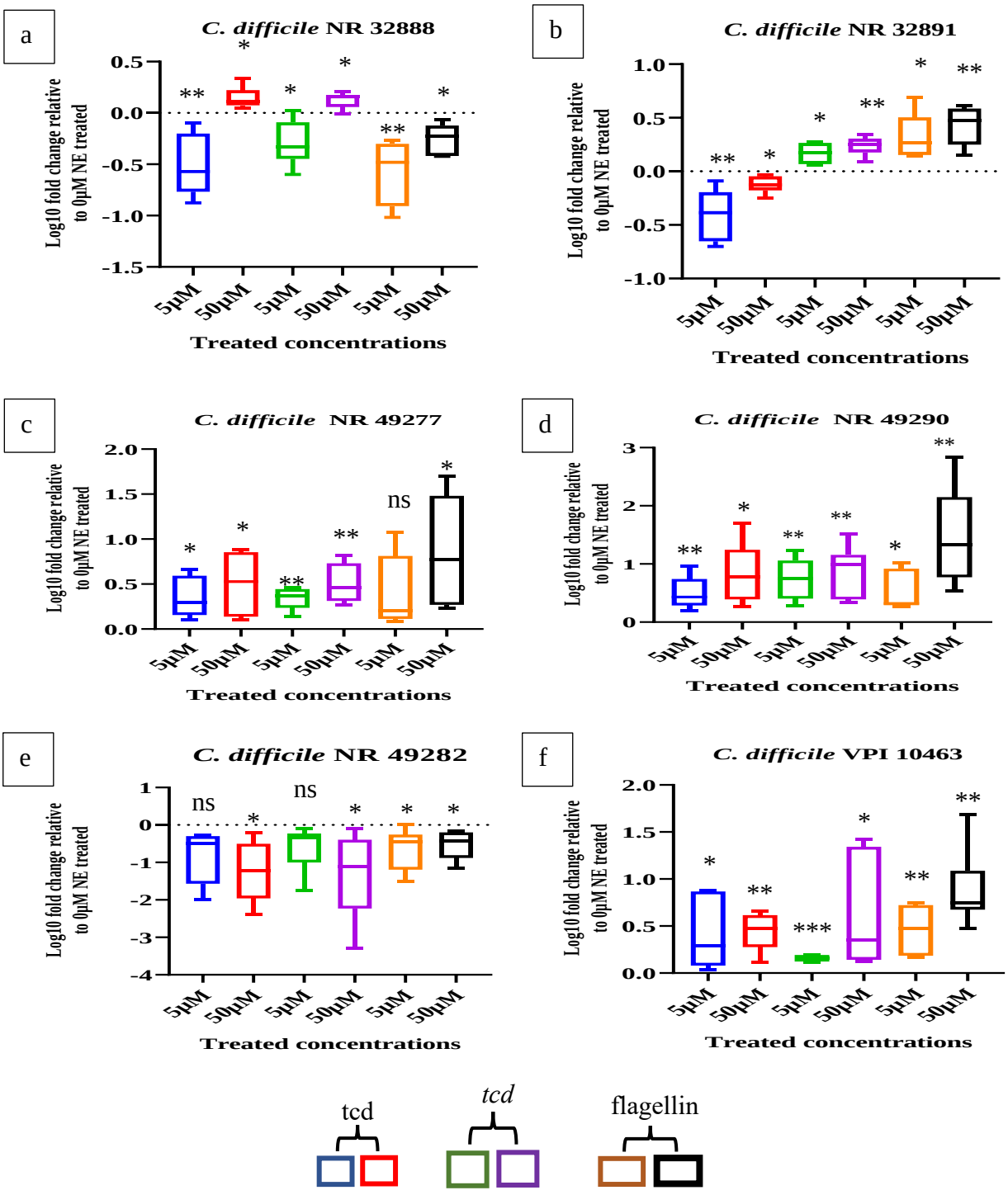


Figure 4.2: Fold change in the virulence-associated gene expression (*tcdA, tcdB, flagellin)* profiles of *C. difficile* strains after treatment with 5 & 50 µM NE. Virulence- associated gene expression levels were analyzed by qPCR and normalized to the reference gene *RpoC*. Based line zero indicated the untreated control. Asterisks indicate a significant difference when compared to untreated (* $p<0.05$,** $p < 0.01$, *** $p<0.001$; ns: $p > 0.05$)

4.4 Effect of NE on the pilin gene of *C. difficile*:

The expression of three pilin genes (*pilA1*, *pilA3*, *pilA5*) in the presence of two different concentrations of NE were analyzed. All three pilin genes were upregulated in NR 49277 (*pilA1*, *pilA3*, *pilA5;* figure 4.3b). In NR 32891 (figure 4.3 a) & VPI 10463 (figure 4.3 d) strains, *pilA1* and *pilA3* genes were upregulated. In NR 49290 (figure 4.3 c), *pilA1* & pilA5 were upregulated.

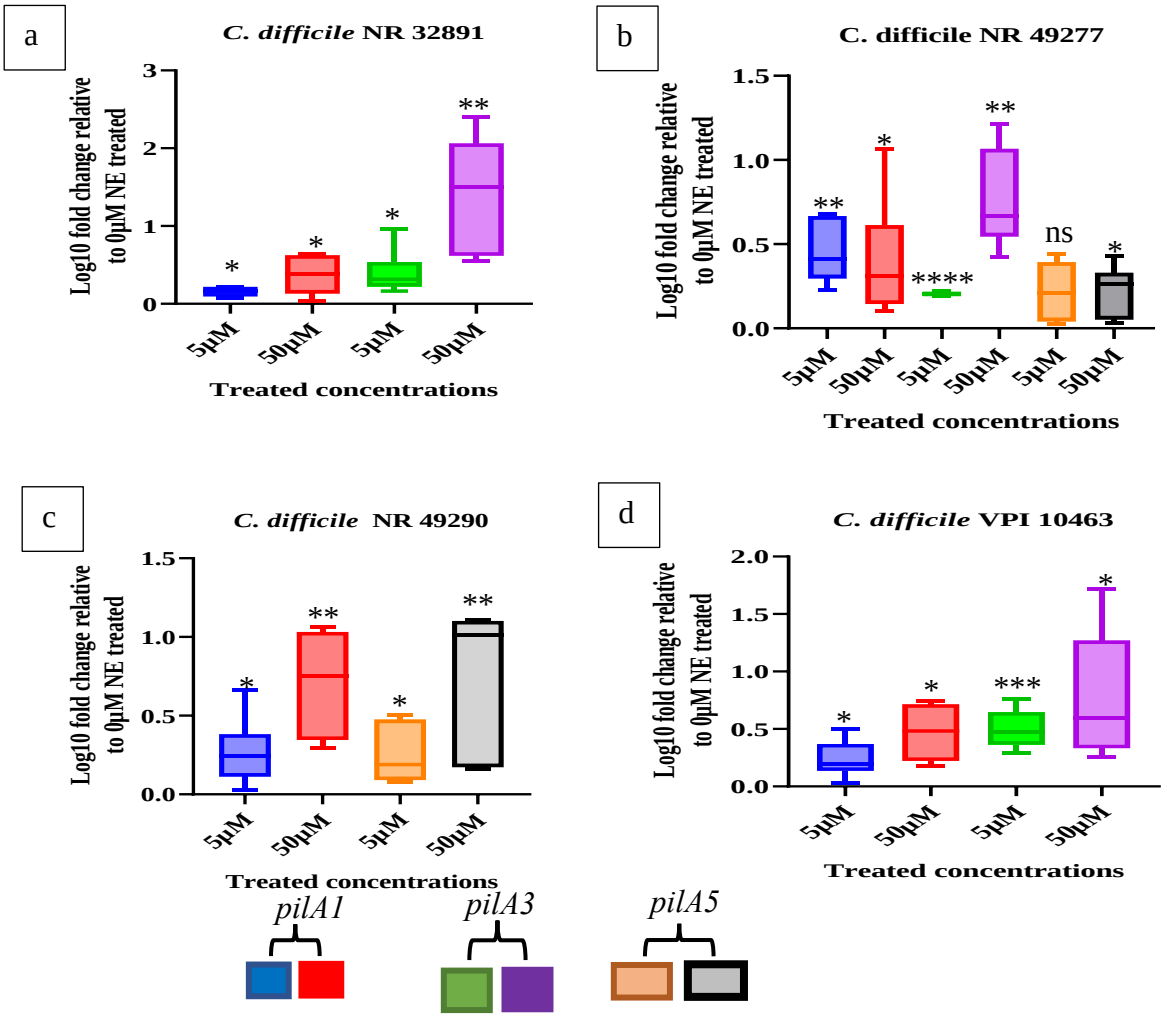


Figure 4.3: The impact of NE in the Pillin genes expression of *C. difficile* (NR 32891, NR 49277, NR 49290 & VPI 10463) after treatment with 5 & 50 μM NE for 8 hours. Based line zero indicated the untreated control. Asterisks indicate a significant difference when compared to untreated (* $p<0.05$,** $p < 0.01$, *** $p<0.001$, **** $p<0.0001$; ns: $p > 0.05$).

However, when compared between the expression of all genes in each strain, the highest upregulation was observed for *pilA1* in NR49290 (50µM treated ~7 fold-P<0.01), *pilA3* in NR 32891 (50µM treated ~14 fold-P<0.001), and *pilA3* in VPI 10463 (50µM treated ~7.7 fold-P<0.05).

For strains NE 32888 *pilA1 & pilA3* gene expression in lower concentration and NR 49282, *pilA1 & pilA3* expression in both concentrations of NE followed an inconsistent pattern in two biological replications.

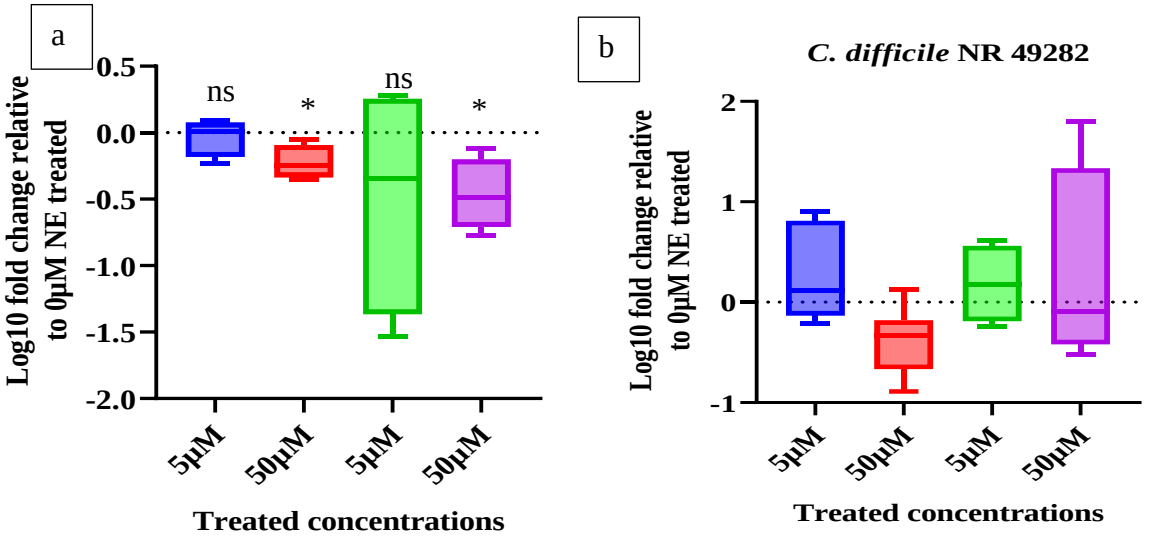
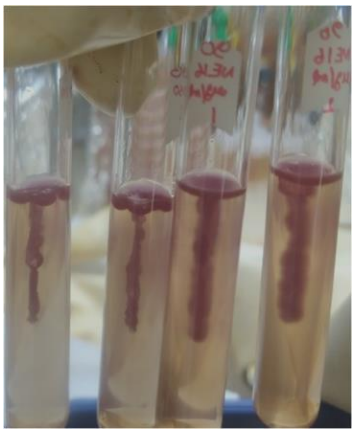


Figure 4.4: The impact of NE in the pilin genes expression of *C. difficile* NR 32888, NR 43282 after treatment with 5 & 50 µM NE for 8 hours. Based line zero indicated the untreated control. Asterisks indicate a significant difference when compared to untreated (* p<0.05, **p < 0.01, ***p<0.001, ****p<0.0001; ns: p > 0.05).

4.5 Effect of NE on the motility of *C. difficile*:

For the motility assay, NR 49290, ATCC BAA 1870 & VPI 10463 were used . In this assay, each 50 µM NE treated strain compared with their respective control tubes (equal volume of DMSO). Results were recorded after 8 hours of incubation. Treatment with NE resulted in increased motility of NR 49292, ATCC BAA 1870 compared to the untreated control.



Control Sample

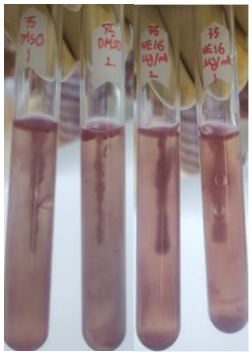
control Sample

Figure 4.5: Image of motility of *C. difficile* NR 49290 & ATCC BAA 1870 after 8hour incubation.

For strain VPI 10463, at 8 hours of incubation, no change was observed, whereas, at 14 hours, enhanced motility was observed compared to the untreated control.

Control Sample

Figure 4.6: Image of motility of C. difficile VPI 10463 after 8-hour incubation



Control Sample

Figure 4.7: Image of motility of C. difficile VPI 10463 after 14-hour incubation

4.6 Effect of NE on anticlostridial agent:

The antibacterial activity of metronidazole and fidaxomicin was tested against *C. difficile* in the presence and absence of NE. Norepinephrine (NE), is the stress-associated neuroendocrine hormone that can modulate bacterial behavior. As a result, bacteria can increase their nutrient

uptake, metabolisms, replication etc. In our microdilution assay, we found that Fidaxomicin is more effective in the presence of NE compared to Metronidazole.

Table 4.1: The minimum inhibitory concentrations (MICs, µg/mL) of Metronidazole and Fidaxomicin against *C. difficile* in the presence and absence of NE

Strain	***C. difficile* NR 49290**		***C. difficile* VPI 10463**	
Anticlostridial drug	**Metronidazole (µg/ml)**	**Fidaxomicin (µg/ml)**	**Metronidazole (µg/ml)**	**Fidaxomicin (µg/ml)**
NE 0 µM	1	0.125	0.25	0.0625
NE 5 µM	0.5	0.125	0.25	0.03125
NE 50 µM	0.5	0.0625	0.25	<0.015

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CHAPTER 5. DISCUSSION

Clostridioides difficile, an anaerobic bacterium, is a common inhabitant of the gut microflora. When the levels of gut bacteria become imbalanced, this benign resident could multiply and cause life-threatening diarrhea. Thus, *Clostridioides difficile* is responsible for antibiotic-associated diarrhea whenever there is an imbalance in the gut microflora associated with the overuse of antibiotics. Community-acquired CDI has been reported in young, healthy individuals even without hospital or antibiotic exposure [2-5]. This drastic change in behavior of *C. difficile* is attributed to the emergence of the hypervirulent strains. These hypervirulent strains express several virulence factors such as toxins (*tcdA* and *tcdB*). Toxins are released in all their growth phases and, as a result, cause severe infections, more extended hospital stays, and death in affected patients [6]. These strains also produce another toxin known as *C. difficile* binary toxin, CDT, which plays a critical role in the recurrence of CDI. They can produce more spores than non-hypervirulent ones [7,8], thus increasing the recurrence rates and more outbreaks of both hospital- and community-acquired infections [9-11]. These spores can remain dormant in the GI tract and later germinate at the end of anticlostridial therapy, leading to recurrent infections. As a result, hypervirulent strains are considered one of the major risk factors for recurrent CDI [6,12].

Long-term hospitalization and treatment trigger stress for patients and stimulate the secretion of stress-associated neuroendocrine hormone NE to mediate stress [1]. As a result, the level of NE is increased in the gut. . The increased level of NE could affect the gut microbiome and modulate the bacterial behavior of several Gram-positive and Gram-negative bacterial species [4-7], but not in *C. difficile*.

In this study, we investigated the effect of the stress hormone NE on the growth and gene expression of specific virulence factors of *C. difficile*.

For investigating strain-specific response, six different strains of *C. difficile* were used. All toxicogenic *C. difficile* strains were obtained from the American Type Culture Collection (ATCC) and Biodefense and Emerging Infections Research Resources Repository (BEI Resources). *C. difficile* NR -32888, NR 32891 are Ribotype 027 and NR 49277, NR 49290, NR 49282 are Ribotype 019 and VPI 10463 is Ribotype 087.

In our study, we used BHI supplemented with yeast extract and L-cysteine for proper growth of *C. difficile*. Cysteine maintained low redox conditions during *C. difficile* cultivation [14,15]. Usually, it is common to use cysteine to prepare pre-reduced culture media for anaerobic bacteria [16] to protect cells from oxidative stress. Another important fact is its low toxicity [17]. Yeast extract contains different nutrients such as vitamins, lipids, amino acids, peptides, and minerals with a balanced composition that can enhance bacterial growth [18]. BHI is suitable for the growth & sporulation of *C. difficile* strains [19,20]. Usually, 37^0C & pH 7.4 ± 0.2 were maintained for optimum growth and toxin production. Lower pH could reduce growth, sporulation & motility [21].

Earlier studies had mentioned the influence of NE on the growth of different microbiomes [6-10]. In our study, we tried to investigate the effect of NE on the growth of *C. difficile*. For this purpose, we treated 6 strains with 0, 5 & 50μM for 8 hours. Three biological replicates were used (each concentration had three or more technical replicates). For observing the density of the bacterial cells, every hour OD was measured at 600nm. In the case of the growth curve, the difference between means of the treated and untreated group was explored by two-way ANOVA followed by Tukey's multiple comparison test. However, significant differences were not observed in growth between the groups with 0 μM NE (control) and 5 μM NE treated bacteria. Moreover, the strain NR 49290, VPI 10463, NR 32888 & NR 32891 was not significantly increased after treatment with 50 μM NE compared to the control. But an exception was observed in the case of NR 49277 & NR 49282. The addition of 50 μM NE significantly stimulated the growth of NR 49277 after 6 hours and continued till 8 hours ($p<0.0001$) compared to 0 μM NE. But in the case of strain NR 49282, 50 μM NE considerably decreased growth at a 7-hour timepoint ($p <0.01$) compared to 0 μM NE, though, in all strains, there was no difference in cell density between control & 5μM NE treated media.

We compared our findings with *Aeromonas hydrophila* , a Gram negative, rod shaped bacteria. It can survive in an aerobic environment. Like *C. difficile,* it can also survive in anaerobic conditions. In the case of *Aeromonas hydrophila*, a significant difference was not present in cell density among 0 and 50 μM NE treated groups even 72-hour incubation [22] which is like our findings though incubation time was higher than ours (8 hours). *Vibrio harveyi* which is a Gram negative , facultatively anaerobic. In the case of *Vibrio harveyi* maximum turbidity was found 1.6-fold higher

when treated with 50 μM NE compared to untreated cultures [23]. However, we observed higher turbidity in strain NR 49277 only while treated with 50 μM NE after 6 hour incubation. In addition, incubation period was higher in *Vibrio harveyi* (16-48 hour) than in our treatment procedure (8 hour). Moreover, the neuroendocrine hormone norepinephrine increases the growth of *Pseudomonas aeruginosa* PA14 (strict aerobe) while treated with 50 μM NE [8]after 8 hours of incubation as like as strain NR 49277. On top of that, the growth of *K. pneumoniae* in the presence of norepinephrine was increased by about 2 to 4 logs ($p < 0.0001$) over 72 hours, the growth of *E. coli* 3logs during the first 48hr growth period ($p < 0.0001$) and a slight increase over time of *S. aureus* growth cultured in the presence of 100 μM norepinephrine [11] compared to 0 μM NE treated. In addition, growth of the obligate anaerobe *Campylobacter jejune* increased after 100 μM norepinephrine treatment (~ 5 hours later) for 50 hours [24] which is similar to our findings in the case of strain NR 49277 (cell density was increased from 6 hour time point to 8 hour) though concentration (50 μM) was lower compared 100 μM norepinephrine treatment.

Fusobacterium nucleatum is an anaerobic bacterium as *C. difficile*. In a research study, it was found that the growth of this bacteria (ATCC 10953, ATCC 25586) is reduced ($p<0.05$) while treated with 50 μM NE [25]. In addition, the growth of anaerobic *Prevotella intermedia* (ATCC 25611), *Porphyromonas gingivalis* (FDC381, ATCC 33277), *Tannerella forsythia* (ATCC 43037) were also reduced ($p<0.05$) after 50 μM NE treatment [25]. Besides, several bacterial species under *Actinomyces* genus group (*Actinomyces israelii* ATCC 12102, *Actinomyces viscosus* ATCC 43146) showed reduced growth ($p<0.05$) after incubation with 50 μM NE [26]. In this study, the incubation time was longer than our procedure(12-24 hour), and all are anaerobic bacteria like our experimental strain. Similar findings we got in our study in the case of strain NR 49282. Growth of strain NR 49282 is reduced ($p<0.01$) after 7 hours of 50 μM NE treatment.

C. difficile causes it's pathogenicity by the expression of pathogenicity locus (PaLoc; 19.6kb). In this PaLoc, *tcdA* gene which encode toxin A (*toxA)* along with *tcdB* genes are situated [27,28]. However, the nonvirulent strain, instead of PaLoc, a fragment containing 127 base pairs is found in that position [29]. *toxA* (308 kDa) the largest toxins [30]. This toxin attacks human intestinal epithelial cells (IECs) and disrupts the intestinal linage [31,32]. Primarily, toxA attacks tips of the intestinal villi, which leads to cell membrane erosion and fluid leakage. Finally, the symptoms of pseudomembranous colitis appear due to cell erosion and apoptosis [33].

Our study found that the toxin genes- *tcdA* is upregulated in NR 49290, NR 49277 & VPI 10463 strains in both concentrations of NE. While treated with 5 & 50μM NE, exotoxin *tcdA* of NR 49290 (~5.6 fold-P<0.01& ~8.3 fold-P<0.05), NR 49277 (~3.5 fold-P<0.05, ~5 fold-P<0.05) & VPI 10463 (~4.1 fold-P<0.05& ~4.5fold- P<0.01) significantly increased. In addition, *tcdA* gene is down-regulated in the other 2 strains: NR 49282& NR 32891, in both NE treatments. For strain NR 49282 (~4 fold- P<ns & ~12 fold-P<0.05) & NR 32891 ((~4 fold- P<0.01 & ~1.2 fold-P<0.05) reduced expression was notified after 8 hours treatment with 5 & 50μM NE. The exception is found in the case of strain NR 32888. Concentration-specific up and down-regulation were observed; 5 μM(~5.1 fold-P<0.01) treatment caused reduced expression, but 50 μM treatment was responsible for increased expression (~1.4 fold-P<0.01).

ToxB is another toxin among two major toxins. This cytotoxin (270kDa) [34] shows its cytotoxic activity by damaging the tight junction of the cell. However, the most toxic effect is found in human cervical epithelial cells [35]. ToxB affects the cytoskeleton of the targeted cell, which leads to an interruption in signal transduction and maintaining the shape of the cell [36]. Principally, ToxB increases the quantity of cytosolic actin, which is the soluble form of the actin filament. As a result, cells cannot form tight round shapes to maintain their integrity [37]. In our experiment, *tcdB* expression is significantly increased in both concentration in NR 49290 (~7.4 fold -P<0.01& ~8.8 fold-P<0.01), NR 49277 (~3.1 fold-P<0.01 & ~5.4 fold- P<0.01), VPI 10463 (~1.5 fold P<0.001& ~6.2 fold-P<0.05), NR 32891 (~ 1.6 fold-P<0.05, ~2.3 fold-P<0.01). Besides, reduced in NR 49282 (~1.6 fold- P<0.05 & ~2.3 fold-P<0.01) respectively. However, in the case of strain NR 32888, concentration-specific up and down-regulation was observed in *tcdB* as the previous *tcdA*; 5 μM(~2.9 fold-P<0.05) treatment caused reduced expression, but 50 μM treatment responsible for increased expression (~1.2 fold P<0.05). NE also increases the pathogenicity of other bacteria by increasing toxin production. For instance, the production of Shiga-like toxins (SLT-I,II) by *E. coli* is influenced by NE, such as increasing over 100-fold (SLT-I) after 24 hours and 50-fold (SLT-II) after 12-hour incubation [38].

For motility, colonization, and adherence, bacteria use the flagellin gene. NE increases the motility of bacteria such as *Vibrio harveyi* [10], *E. coli* [7], *Campylobacter jejuni* [24], etc. *C. difficile* causes pseudomembranous colitis through its several virulence factors. Flagellin gene *fliC* is one of the virulence factors. *C. difficile* needs to adhere and colonize to interact with its' targeted cell.

Otherwise, a nonspecific defense mechanism quickly eliminates these bacteria if they cannot attach and colonize the targeted epithelial cells [39]. The *fliC* protein has the adhesive property [40], with a molecular mass of *fliC* protein 39kDa [41].In our study we found that flagellin gene expression upregulated in strain NR 49290 (~5.4fold- P<0.05&~14 fold-P<0.01), VPI 10463 (~4.6 fold-P<0.01 & ~8.8 fold- P<0.01), NR 49277(~4 fold- P= ns & ~ 8.5 fold-P<0.05) & NR 32891 (~3.2 fold-P<0.05 & ~4.2 fold-P<0.01) after incubated with 5 & 50μM NE for 8 hours. Besides, down-regulation observed in NR 49282 (~6.3 fold-P<0.01 & ~5.2)& NR 32888 (~5.7 fold-P<0.01 & ~2.6 fold-P<0.05) under same treatment which quite similar to *fliD* & *fliR* are 2 genes of *E. coli*. *fliD* is responsible for cell motility and is the flagellar biosynthesis protein. While treated with 50 μM NE, *fliD* 1.4 fold & *fliR* 2.3 fold increased [7]. In addition, in *Vibrio harveyi,* flagellar motility related genes *flaA, flaC, flaK, fliA, fliS, fldB, cheA, cheR, lafA & lafR* ~1.6-2.2 fold increased after 50 μM treatment which is lower than *fliC* of *C. difficile* under the same treatment.

C. difficile produces multiple toxins responsible for causing chronic diarrhea and pseudomembranous colitis. For this purpose, *C. difficile* binds to its host cell and secretes toxin through a type IV secretion system (T4SS) [42, 43]. In this secretion system, bacteria create an extracellular structure with an injector to transfer toxins [44]. Different pilin proteins are required for the formation of T4SS. The principal subunit of T4SS is *PilA1*. *PilJ* is the minor pilin [45]. *PilA3, PilA5* and *pilA6* are other pilin proteins. Pilin gene also involves bacterial pathogenicity through motility. Previously it was observed that NE induced transcriptional activator of Pilin gene in *E.coli* [46]. *rfaH* (F pillin) gene of *E. coli* was 1.4-fold increased after NE treatment. We investigated pilin gene expression in our experiment. Interestingly, Pillin genes were consistently upregulated in NR 49277 (*pilA1, pilA3, pilA5*), NR 49290 (*pilA1, pilA5*), VPI 10463 & P13 (*pilA1, pilA3*) strains. Highest upregulation notified, *pilA1* in Strain NR49290 (50μM treated ~7 fold-P<0.01), *pilA3* in P13 (50μM treated ~14 fold-P<0.001) and *pilA5* in VPI 10463(50μM treated ~7.7 fold-P<0.05).

Though pilin gene expression was increased and consistent in other studies, NR 32888 and NR 49282 were quite different. In toxicogenic strain, NR 32888 pilA1, pilA3 was not giving similar expression in 5μM NE treated 2 biological replicates, although 50μM NE treatment downregulated in both biological replicates. Interestingly NR 32888 & NR 32891 are toxicogenic, isolated from the fecal sample, but the pilin gene expression pattern is quite different. Besides, NR 49282

expression was different than other strains (NR 49277, NR 49290) belonging to the same Ribotype group 019. In NR 49282, toxin & flagellin genes were down-regulated in both concentrations of NE treatment, and pilin genes *pilA1, pilA3* expression was not similar in its biological replicates.

A motility test assay was performed using NR 49290 ribotype 019, ATCC BAA 1870 ribotype 027 & VPI 10463 ribotype 087 in semisolid media. All are toxin-producing pathogenic strains. After 50μM NE treatment for 8 hours, motility was increased in NR 49290, ATCC 1870. But for VPI 10463, motility was not significantly changed till 8 hours. A significant change was visualized after 14-hour incubation.

For assessing the anticlostridial activity in the presence of NE, Metronidazole & Fidaxomicin were used. In higher concentrations, Fidaxomicin was more effective than Metronidazole. Usually, NE modulated bacterial metabolism. Increased concentration of NE initiates more uptake of the drug and nutrients. Fidaxomicin was more effective than another one.

In conclusion, it was found that in a short period, NE increases the growth of in *C. difficile* strain (NR 49277) compared to other bacteria such as *Aeromonas hydrophila, Vibrio harveyi*, etc, as well as decreases the growth (NR 49282) compared to *Fusobacterium nucleatum, Prevotella intermedia, Tannerella forsythia, Actinomyces israelii, Actinomyces viscosus* etc. However, in the other 4 strains, growth was not increased. Though strain-specific up or down or no influence was observed after NE treatment, it could be concluded that virulence gene expression was influenced in all strains. Besides, 50μM NE treatment regulates mostly virulence gene expression. For *tcdA, tcdB & fliC* gene highest upregulation observed (~8.3 fold-$P<0.05$,~8.8 fold-$P<0.01$ & &~14 fold-$P<0.01$) in strain 49290 after 50μM NE treatment. For pilin gene expression, the highest expression *pilA1* in Strain NR49290 (~7 fold-$P<0.01$). But *pilA3* in P13 (~14 fold-$P<0.001$) and *pilA5* in VPI 10463 (~7.7 fold-$P<0.05$) highly expressed.

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